





## NONTOXIC MUCOSAL ADJUVANT.

FIELD OF THE INVENTION

5       The present invention relates to an adjuvant useful for the administration of vaccines to organisms. In particular, the adjuvant of the invention allows the delivery of vaccines to mucosal surfaces to raise a secretory and systemic immune response.

10

BACKGROUND TO THE INVENTION

Current vaccination technology is based almost exclusively on systemic vaccination techniques wherein the vaccine is injected into the subject to be vaccinated. Only certain live/attenuated vaccines, such as the Sabin polio vaccine, may be taken orally.

20       The advantages of oral immunisation techniques are several fold. For instance, it is self-evident that a vaccine which may be fed to subjects is easier to administer on a large scale in the absence of specialised equipment, especially to subjects which may be difficult to handle or even locate, such as livestock and wild animals. The spread of infection by the re-use of needles in developing countries would thereby be avoided. Furthermore, an oral vaccine may be provided in the form of an edible solid, which is easier to handle under extreme conditions and is more stable than liquid suspensions as currently used.

30       Moreover, delivery of immunogens to a mucosal membrane, such as by oral or intranasal vaccination, would permit the raising of a secretory immune response.

The secretory immune response, mainly IgA-mediated, appears to be substantially separate from the systemic immune response. Systemic vaccination is ineffective for raising a secretory immune response. This is a considerable disadvantage when considering immunisation against pathogens, which often enter the subject across a mucosal surface such as the gut or lung.







of these publications conclude that there is a link between the ADP ribosylating activity of CT and/or LT and the adjuvant activity. It appears from these publications, therefore, that CTB or a non-toxic mutant of CT or LT would not be active as a mucosal adjuvant.

#### SUMMARY OF THE INVENTION

There therefore remains a need for an active mucosal adjuvant which may be used to increase the immunogenicity of an antigen when administered to a mucosal surface, such as orally or intranasally.

It has now been discovered that, in complete contradiction with the results and conclusions presented in the prior art, the toxic and adjuvant activities of the ADP-ribosylating toxins are separable. An entirely non-toxic mutant of such a toxin has been shown to be active as a mucosal adjuvant.

The present invention, in a first aspect, provides a pharmaceutical composition comprising a non-toxic mucosal adjuvant in admixture with a second antigen.

It has been demonstrated that an LT mutant which completely lacks toxicity is active as a mucosal adjuvant and protects subjects against subsequent challenge with a lethal dose of the immunogen. Although the Applicants do not wish to be bound by any particular theory, it is postulated that the results of Lycke et al. and Holmgren et al. quoted above may be contradicted at least in part because they fail to take into account the stability of the mutant being made. Inter alia by ensuring that the non-toxic mutant of the invention is stable at the site of delivery, it has been demonstrated that the adjuvant effect of CT and/or LT may be maintained while its toxic effects are eliminated.

Preferably, therefore, the non-toxic mucosal adjuvant is a detoxified mutant of a bacterial ADP-ribosylating toxin, optionally comprising one or more amino acid additions, deletions or substitutions.

Particularly suitable are detoxified mutants of CT or LT. For example, a mutant LT in accordance with the invention may possess an Arg7 to Lys7 substitution at position 7 of the A subunit, the so-called LTK7 mutant.

5 Alternative mutants are known to those skilled in the art and are preferred molecules for use in the present invention. Examples include PT mutated at position 129, in particular PT having a Glu 129->Gly mutation. Further mutants include PT mutated at one or both of Trp 26 and Arg  
10 9, optionally in combination with the Glu 129 mutation.

The mutant used in the invention may moreover be a mutant wherein the mutation has been effected in a part of the molecule which results in the prevention of proteolytic cleavage of the A subunit of the toxin, such that enzymatic  
15 activity is not brought about. Such mutants are described in Grant et al. Inf. and Immunity (1994) 62(10) 4270-4278. For example, the mutant may comprise an Arg 192->Gly mutation in LT or a corresponding mutation in another ADP-ribosylating toxin.

20 The mutant of the invention is preferably in the form of a holotoxin, comprising the mutated A subunit and the B subunit, which may be oligomeric, as in the wild-type holotoxin. The B subunit is preferably not mutated. However, it is envisaged that a mutated A subunit may be  
25 used in isolation from the B subunit, either in an essentially pure form or complexed with other agents, which may replace the B subunit and/or its functional contribution.

Methods for the design and production of mutants of  
30 CT and/or LT are known in the art. Suitable methods are described in International Patent Application WO93/13202 (Biocine Sclavo), the disclosure of which is incorporated herein by reference, as well as WO92/19265 (Amgen).

The adjuvant of the invention is preferably  
35 administered in admixture with a suitable antigen against which it is desired to raise an immune response. If the antigen and the adjuvant are not in admixture, it is preferred that they be administered within a relatively

short time of each other, at the same site of administration. It has been observed that the adjuvant effect provided by wild-type CT is short lived (see Lycke and Homgren, Immunology 1986; 59: 301-308). In an alternative embodiment, the mucosal adjuvant of the invention may be administered, optionally in isolation from other antigens, as a boost following systemic or mucosal administration of a vaccine.

The precise formulation of the vaccine may vary in accordance with the nature of the immunogen. For example, if the antigen is enclosed in slow-releasing microspheres or liposomes, the mucosal adjuvant may be similarly enclosed so that the antigen and the adjuvant may interact simultaneously with the mucosal immune system. Alternatively, separate mucosal administration of the adjuvant of the invention may be used to enhance mucosal response to parentally-administered vaccines.

In a second aspect, the present invention provides the use of a non-toxic mutant of CT or LT as a mucosal adjuvant in the preparation of a composition for mucosal administration.

Preferably, the composition is a vaccine and is useful for the immunisation of a subject against a disease or the treatment of a subject suffering from a disease.

Preferably, the mutant comprises one or more amino acid additions, substitutions or deletions in the amino acid sequence of the A subunit of CT or LT which is or are effective to abolish the toxicity of the toxin.

According to a third aspect of the invention, there is provided a method for the prevention or treatment of a disease in a subject comprising administering to the subject an immunologically effective dose of an antigen adjuvanted with a non-toxic CT or LT mutant by contacting a mucosal surface of the subject with said adjuvanted antigen.

The mucosal surface may be any suitable mucosal surface of the subject. For example, the administration may be carried out by inhalation, by means of a rectal or vaginal suppository, or a pessary, by feeding or other

buccal administration, by means of an aerosol, by intranasal delivery or direct application to mucosal surfaces. Especially preferred are oral and intranasal administration.

The subject may be any organism susceptible to immunisation. Especially indicated are humans and other mammals such as livestock, pets and wildlife.

Diseases against which the subject may be immunised include all diseases capable of being treated or prevented by immunisation, including viral diseases, allergic manifestations, diseases caused by bacterial or other pathogens which enter through or colonise mucosal surfaces, AIDS, autoimmune diseases such as systemic Lupus Erythematosus, Alzheimer's disease and cancers. Examples of viral infections which may be treated or prevented using the invention include infection by DNA viruses, such as EBV and VZV, and in particular herpesviridae, for example HSV and HCMV, adenoviridae, papovaviridae, such as HPV, hepadnaviridae, such as HBV, infection by RNA viruses, such as picornaviridae, especially poliovirus and HAV, rhinoviruses and FMDV, togaviridae, flaviviridae, coronaviridae, paramyxoviridae, such as RSV, orthomyxoviridae, such as influenza virus, and retroviridae, especially HIV. Vaccination against HCV and HDV is also envisaged.

Examples of bacterial infections suitable for treatment or prophylaxis by the invention include infection with *Helicobacter pylori*, streptococci, meningococcus A, B, and C, *Bordetella pertussis* and chlamydia and trachomatis.

Vaccine formulation suitable for delivery at mucosal surfaces may be prepared as set out hereinbelow, while further formulations will be apparent to those of skill in the art. Suitable administration regimes are, likewise, set out below while modifications of the exemplified values will be apparent to those of skill in the art.

Moreover, the invention provides a mutant of CT or LT which is a non-toxic mucosal adjuvant and a second antigen for simultaneous separate or sequential administration. Simultaneous administration of the adjuvant and the second antigen when combined in a single vehicle, carrier or

particle, as exemplified below, is particularly preferred.

The second antigen may be any antigen to which it is desired to raise an immune response in the subject. Suitable antigens comprise bacterial, viral and protozoan antigens derived from pathogenic organisms, as well as allergens, allogens and antigens derived from tumours and self-antigens. Typically, the antigen will be a protein, polypeptide or peptide antigen, but alternative antigenic structures, such as nucleic acid antigens, carbohydrate antigens, and whole or attenuated or inactivated organisms such as bacteria, viruses or protozoa are not excluded. The invention further provides a method for the manufacture of an adjuvanted vaccine comprising the steps of:

- a) performing site-directed mutagenesis on the A-subunit of a bacterial ADP-ribosylating toxin in order to detoxify the toxin; and
- b) bringing the detoxified toxin into association with a second antigen, such that it functions as a mucosal adjuvant.

Specific examples of antigens useful in the present invention include HSV gD, gB and other glycoproteins; HIV gp120 and other proteins; CMV gB or gH; HCV antigens; HDV delta antigen; HAV antigens; EBV and VZV antigens; B. pertussis antigens and H. pylori antigens.

In general, the second antigen may be the immunogenic component of the vaccine intended for injection. Such vaccines, and the immunogenic components thereof, may be subunit vaccines, whole inactivated or attenuated organisms or polynucleotide vaccines.

The vaccines according to the invention may either be prophylactic (to prevent infection) or therapeutic (to treat disease after infection).

These vaccines may either be prophylactic (to prevent infection) or therapeutic (to treat disease after infection).

Such vaccines comprise antigen or antigens, usually in combination with "pharmaceutically acceptable carriers," which include any carrier that does not itself induce the

production of antibodies harmful to the individual receiving the composition.

Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplet emulsions or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. In preferred aspects of the invention, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore, the antigen may be conjugated to a bacterial toxoid, such as a toxoid from diphtheria, tetanus, cholera, H. pylori, etc. pathogens.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (PCT Publ. No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi<sup>TM</sup> adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox<sup>TM</sup>); (3) saponin adjuvants, such as Stimulon<sup>TM</sup> (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs

(immunostimulating complexes); (4) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA); (5) cytokines, such as interleukins (e.g., IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, etc.), interferons (e.g., gamma interferon),  
5 macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc; and (6) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Alum and MF59 are preferred.

As mentioned above, muramyl peptides include, but are  
10 not limited to, N-acetyl-muramyl-L-threonine-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

15 The immunogenic compositions (e.g., the antigen, pharmaceutically acceptable carrier, and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, etc.

Additionally, auxiliary substances, such as wetting  
20 or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in,  
25 liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect, as discussed above under pharmaceutically acceptable carriers.

Immunogenic compositions used as vaccines comprise an  
30 immunologically effective amount of the antigenic polypeptides, as well as any other of the above-mentioned components, as needed. By "immunologically effective amount", it is meant that the administration of that amount to an individual, either in a single dose or as part of a  
35 series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (e.g., nonhuman primate,

primate, etc.), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and  
5 other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The immunogenic compositions are conventionally administered parenterally, e.g., by injection, either  
10 subcutaneously or intramuscularly. Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories, and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may  
15 be administered in conjunction with other immunoregulatory agents.

Examples of suitable immunostimulatory agents include interleukins, such as interleukins 1,2, 4-7 and 12, and interferons, especially  $\gamma$ -interferon.

20 The invention is described hereinbelow by way of example only, with reference to the following Figures:-

#### DESCRIPTION OF THE FIGURES

25 Figure 1a shows the titre of total ovalbumin specific antibody in BALB/c mice immunised i/n or s/c with either ovalbumin alone or ovalbumin together with toxin derivatives;

Figure 1b shows the titre of total toxin-specific  
30 antibody in the mice of Figure 1a;

Figure 2 shows a measurement of ovalbumin-specific IgA in nasal and lung lavages of mice injected as in Figure 1; and

Figure 3 shows the presence of tetanus toxoid-specific  
35 antibodies in the serum of BALB/c mice immunised i/n or s/c with tetanus toxin fragment C alone or together with toxin derivatives.

DETAILED DESCRIPTION OF THE INVENTION

5        Site-directed mutagenesis was used to replace the arginine residue at position seven of the A subunit of LT with lysine in order to construct a non-toxic LT mutant that could still assemble as a holotoxin with cell binding activity. The mutant protein, named LTK7, was purified and  
10    tested for ADP-ribosyltransferase and toxic activity in several assays. LTK7 was still able to bind GM1 ganglioside receptor but showed a complete loss of enzymatic activity, in agreement with published data (Lobet et al., Infect. Immun. 1991; 59:2870-2879). Further, LTK7 was inactive in  
15    the mouse ileal loop assay and in vitro on Y1 cells, even when a dose equivalent to  $10^7$  toxic units of wild-type LT was tested (Table 1).



Sample bleeds of 100µl were collected on day 0, 21, 35, 56 and on day 76 animals were culled by cardiac puncture.

Quantitation of antibody was estimated by ELISA. For estimation of ovalbumin-specific antibodies, 96-well EIA plates (costar) were coated overnight with 60 µg/ml of ovalbumin. Measurement of toxin-specific antibodies was performed using a GM1 capture ELISA. Toxin-specific antibodies were measured against the antigen used in the immunisations. No single toxin was used in the measurements of toxin-specific antibody from each group, and as such the titres between these groups can not be compared directly.

Sera from each group were pooled from four and two mice respectively. Samples were prepared in duplicate from a dilution of 1:50. Absorbences were read at 450nm using the kineticalc version 2.13 programme (Biotek instruments). This programme calculates the rate of change of substrate over thirty time points ten seconds apart.

ELISA titres of antibody were measured arbitrarily as the dilution of serum which gave half the maximal absorbence at 450nm. Sera which failed to show absorbence at 450nm 2.5 times greater than that observed with the equivalent pre-immune sera were considered negative. Results shown in Figure 1a and 1b represent the mean titre values from duplicate wells from one experiment. No significant levels of antibodies to ovalbumin above background were detected in the serum of mice immunised i/n with ovalbumin alone although mice immunised s/c efficiently sero-converted. Mice receiving ovalbumin along with either CT or LT i/n contained very high levels of anti-ovalbumin antibodies in their sera. These were equivalent to those observed when mice immunised s/c. Mice that received ovalbumin with LTK7 also showed very high levels of antibodies to ovalbumin.

The levels of anti-toxoid responses in these same groups are shown in Figure 1b. All mice, including those immunised with the mutant toxin, developed high levels of antibodies to these toxin in their sera.

The local secretory antibody levels to ovalbumin were

measured using both lung and nose washings (Fig. 2). In brief animals were culled by cardiac puncture and dissected so that the trachea was exposed. An ultra-thin pipette was then inserted into a small nick in the trachea. Lung washes  
5 were collected by repeated flushing and aspiration of 1.5 ml of 0.1% bovine serum albumin (Sigma), in PBS, into the lungs. Nose washes were collected by flushing 1ml of 0.1% BSA in PBS through the nasal cavity.

Ovalbumin-specific IgA antibodies were measured by  
10 ELISA using an anti-mouse alpha-chain-specific conjugate antibody (Serotec). Samples were prepared from individual animals and columns in this figure represent the mean rate of change of substrate, using kineticcalc, for four and two mice immunised i/n and s/c respectively. The figures are  
15 constructed using the raw absorbance data at a dilution of 1:3 with respect to lung washes. These correspond to titres of between 1:2 and 1:6 for nose washes and between 1:70 and 1:120 for lung washes. These titres were calculated using the method described above. Mice immunised s/c or i/n with  
20 ovalbumin alone contained no detectable ovalbumin-specific IgA in the washings sampled. All individual mice immunised with ovalbumin in combination with CT, LT or LTK7, showed detectable levels of anti-ovalbumin IgA. Thus both a local and systemic anti-ovalbumin response are detectable in these  
25 animals.

In the face of these encouraging experiments with ovalbumin the immunisation was repeated using Fragment C, a 50,000 dalton, non-toxic portion of tetanus toxin which had been expressed in and purified from the yeast *Pichia*  
30 *pastoris*. Mice were immunised either s/c or i/n with Fragment C alone or mixed with individual samples of either LT or LTK7. Mice were separated into four groups of ten mice and four groups of five mice. Ten mice were immunised i/n with a) 10µg of fragment C alone; b) 10µg of fragment C  
35 + 1µg of LT; c) 10µg of fragment C + 1µg of LTK7 and d) PBS only, all in a final volume of 30 µl. Five mice were immunised i/n with a) 1µg of LT and b) 1µg of LTK7. The remaining two groups of mice were immunised s/c with either

no protein or 10µg of fragment C in a dose volume of 100µl. These vaccines were prepared as described in Figure 1. Animals were immunised on day 1 and 22. Sample bleeds of 100µl were collected on day 0, 21 and 35. Fragment C-specific antibodies were measured by ELISA using tetanus toxoid (10µg/ml) as the coating antigen. Sera from each group were pooled. Samples were prepared in duplicate from a dilution of 1:50. ELISA titres were calculated as described above. Mice immunised s/c with Fragment C efficiently sero-converted producing high levels of anti-Fragment C antibodies (Fig. 3). Mice immunised i/n with Fragment C alone showed no significant sero-conversion. However mice immunised with Fragment C combined with LT or LTK7 showed high levels of anti Fragment C antibodies in their sera (Fig. 3). Since mice that sero-convert to Fragment C can be protected against toxin challenge the groups were challenged with active tetanus toxin. All mice immunised s/c with Fragment C alone were protected whereas all mice immunised i/n were highly susceptible. All mice i/n immunised with Fragment C combined with either LT or LTK7 survived the challenge (Table 2).

TABLE 2

	Serum anti-Fragment C	Deaths
LT	---	10/10
LTK7	---	10/10
LTK7 + Fragment C	++	0/10
Lt + Fragment C	++++	0/10
Fragment C	+/-	10/10

The titre of anti-Fragment C antibodies in the serum of mice was on average about 1/3,000 in mice vaccinated with the K7 mutant + Fragment C and 1/12,000 for LT + Fragment C.

These experiments show that protective immunity against tetanus can be achieved using a non-toxic LT mutant as adjuvant and that mucosal immunisation with this molecule can generate both local secretory and systemic immune response to the toxin and co-administered bystander antigens.

CLAIMS:

1. A pharmaceutical composition comprising a non-toxic mucosal adjuvant in admixture with a second antigen.
- 5 2 A composition according to claim 1 wherein the non-toxic mucosal adjuvant is a detoxified mutant of a bacterial ADP-ribosylating toxin.
3. A composition according to claim 2 wherein the non-  
10 toxic adjuvant is a detoxified mutant of CT or LT.
4. A composition according to claim 2 or claim 3 wherein the non-toxic mucosal adjuvant comprises one or more amino acid additions, deletions or substitutions in the A subunit  
15 of the holotoxin.
5. A composition according to claim 4 wherein the non-toxic mucosal adjuvant is LTK7.
- 20 6. Use of a non-toxic mutant as defined in any one of claims 2 to 5 as a mucosal adjuvant in the preparation of a composition for mucosal administration.
7. Use according to claim 6, wherein the composition is  
25 a vaccine.
8. Use according to claim 7, wherein the vaccine is for use in prophylactic or therapeutic applications.
- 30 9. Use according to any one of claims 6 to 8, wherein the composition further comprises a second antigen.
10. A method for the prevention or treatment of a disease in a subject comprising administering to the subject an  
35 immunologically effective dose of an antigen adjuvanted with a non-toxic mutant as defined in any one of claims 2 to 5 by contacting a mucosal surface of the subject with said adjuvanted antigen.

11. A method according to claim 10 comprising administration of a composition according to any one of claims 1 to 5.

5 12. A method according to claim 11 or claim 12, wherein the adjuvanted antigen is administered orally or intranasally.

13. A non-toxic mucosal adjuvant and a second antigen for  
10 simultaneous separate or sequential administration.

14. A non-toxic mucosal adjuvant and a second antigen for simultaneous administration when combined in a single vehicle, carrier or particle.

15

15. A method for the manufacture of an adjuvanted vaccine comprising the steps of:

- a) performing site-directed mutagenesis on the A-subunit of a bacterial ADP-ribosylating toxin in order to  
20 detoxify the toxin; and
- b) bring the detoxified toxin into association with a second antigen, such that it functions as a mucosal adjuvant.





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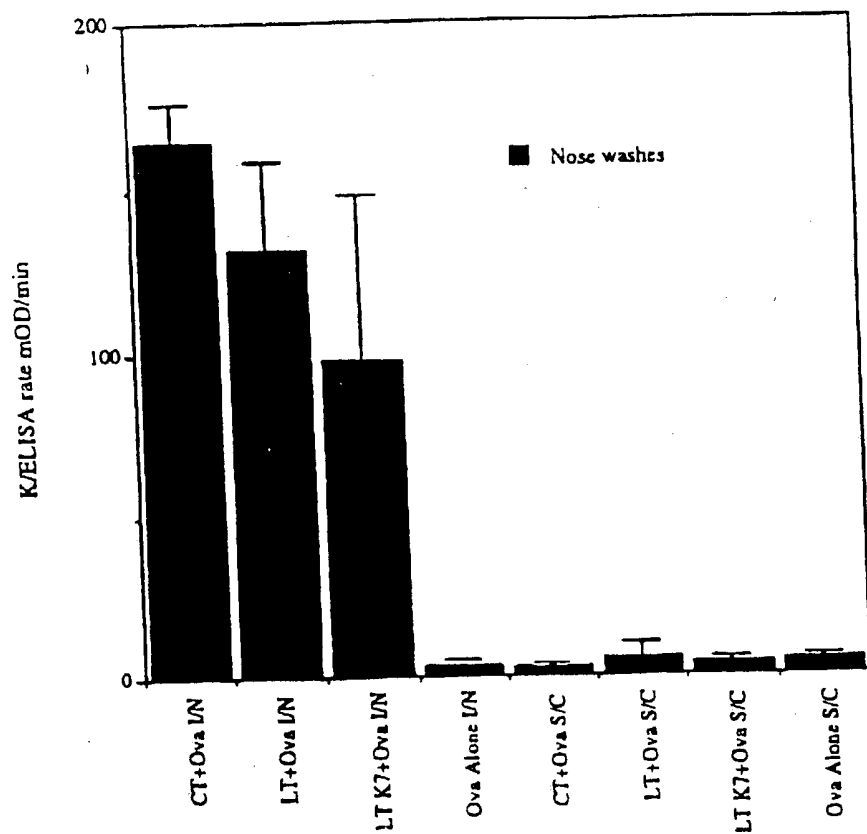


FIG. 2(i)

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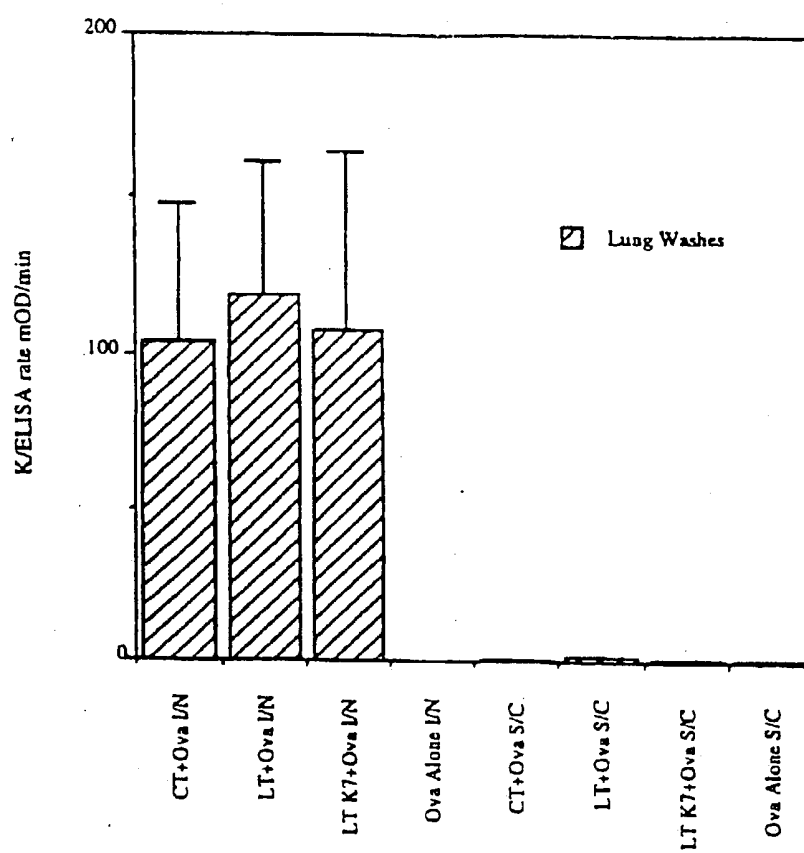


FIG. 2(ii)

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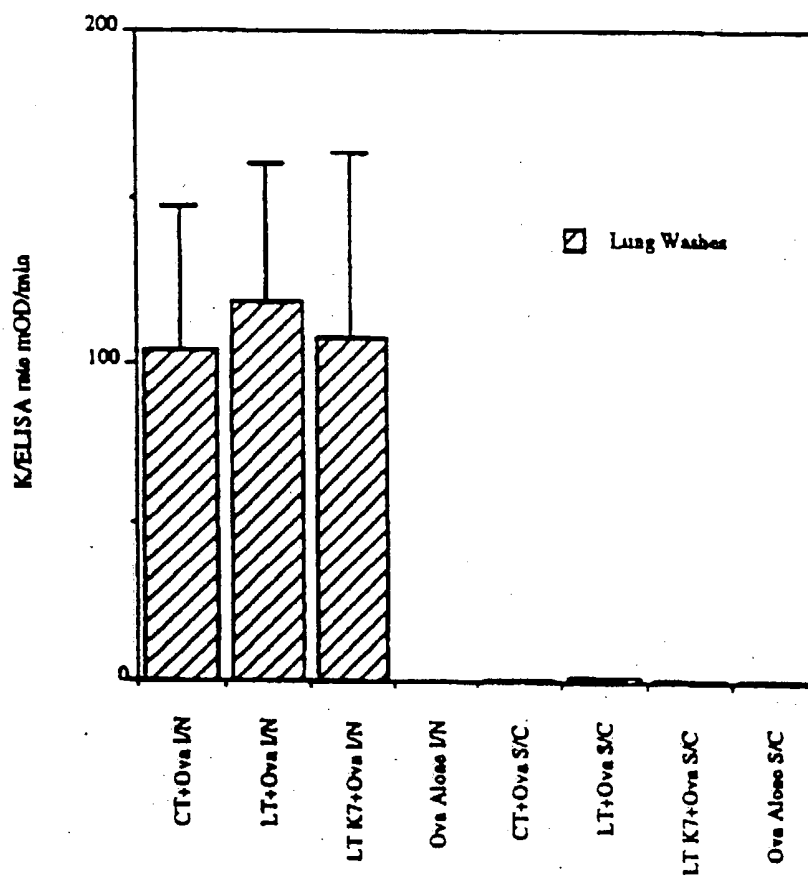
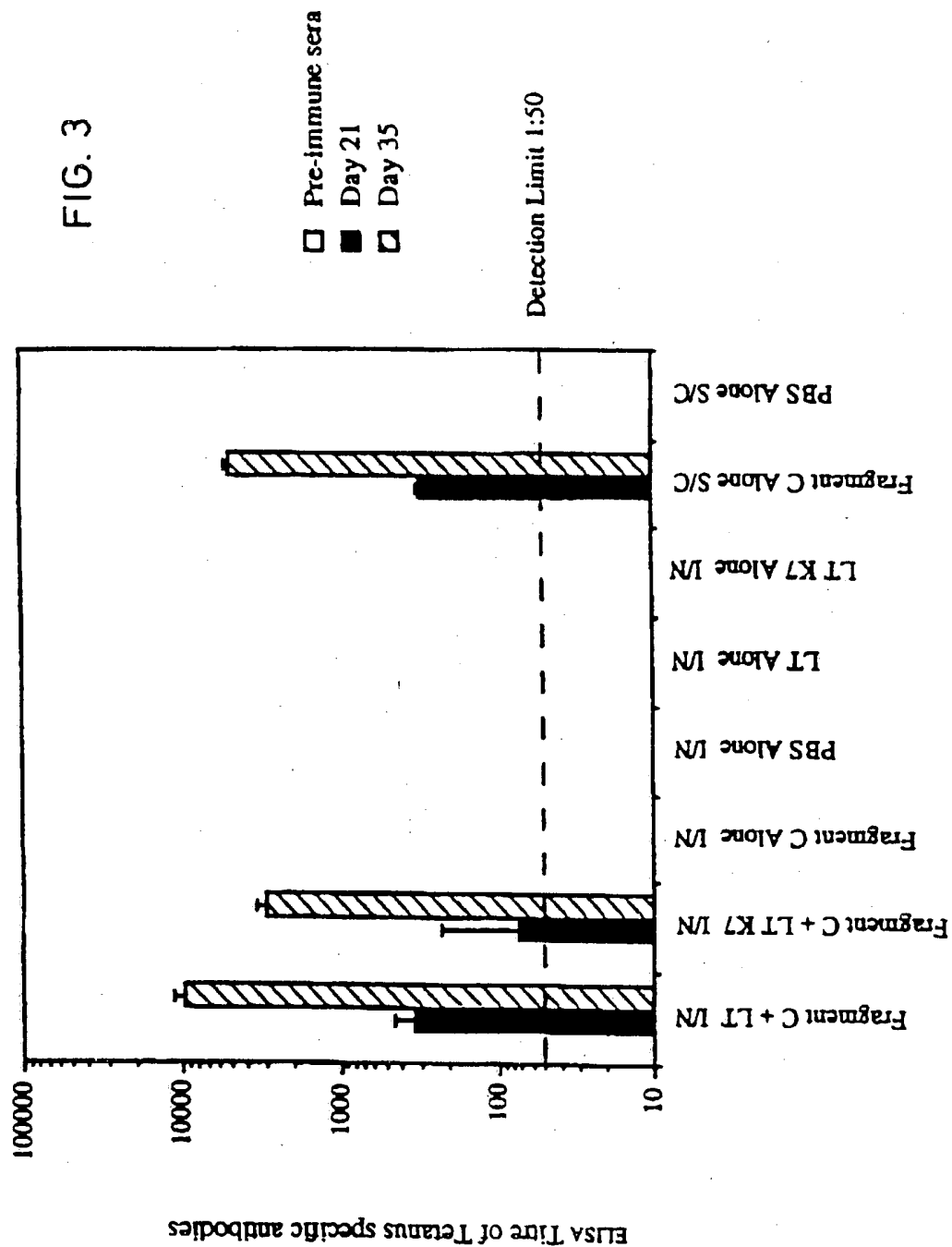


FIG. 2(ii)

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FIG. 3



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